Novel Therapeutic Extracts and Molecules for Degenerative conditions

Field of the Invention

The present invention relates to novel therapeutic plant extracts and molecules for the treatment of degenerative diseases like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye and having antioxidant action.

According to the present invention plant extracts derived from common plants have been characterized using a) chemical fingerprinting by HPLC and b) etiology based in-vitro cell assays in order to reduce the extracts to minimum constituent to use for therapeutic purpose against degenerative diseases like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye and having antioxidant action. The extracts have been chemically fingerprinted and have gone through assays to screen anti-diabetic activity. Specific leads that have shown very specific anti-diabetic activity.

However the present invention also includes testing these extracts in other assays, for generating information of there applicability in the prevention or treatment of degenerative conditions as mentioned above.

Background of the Invention

Degenerative diseases develop over a period of time, with the symptoms and signs becoming progressively worse and the affected person's life becoming increasingly and generally irreversibly affected.

Although any part of the body can be involved, three systems in particular are prone to debilitating degenerative diseases - the nervous system, the muscular system, and the skeletal system.

The classification of degenerative disorders of the nervous system is difficult and somewhat arbitrary. Many of these conditions are familial or hereditary, breed-related, and involve degeneration of the nervous system within the first few months after birth. Premature degeneration of any component of the CNS, such as neurons, myelin sheaths or axons, can be considered under the broad panoply of abiotrophies which are disorders associated with an inherent lack of vital trophic or nutritive factor(s).

Disorders of muscle function include weakness, cramps (painful spasms), and degenerative changes amongst other problems.

Diabetes is often defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by insulin, which leads to abnormalities in the assimilation of carbohydrates in the body. This results primarily in elevated fasting and post-prandial blood glucose levels. If this imbalanced homeostasis does not return to normalcy and continues for a protracted period of time, it leads to a condition known as hyperglycemia that will in due course turn into a syndrome called Diabetes mellitus. Diabetes mellitus can be defined as a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins along with increasing risks of complication from vascular diseases. Very often, the seriousness of the disease is realized on the development of secondary symptoms that manifest in many forms, namely, difficulty in healing of wounds, neuropathy and so on. It is estimated currently, that over 143 million people all over the world suffer from Diabetes mellitus and in the case of most people, suffering from Diabetes; it is not properly diagnosed until irreversible complications set in. The long terms complications arising out of untreated or ineffectively treated Diabetes include, cardiovascular diseases and strokes, retinopathy, nephropathy and peripheral neuropathy.

The World Health Organization has projected India, as the country with the fastest growing population of diabetics and it is estimated that between 1995-2025, Diabetes patients in India will increase by 195%.

In order to maintain the blood glucose level within the normal range, diabetes mellitus is frequently treated using a combination of diet therapy with ergotherapy, insulin therapy, pharmaceutical therapy and so on. The major pharmacological agents available to lower blood glucose are – insulin seceretagogues and sensitizors.

Insulin Sensitizers are molecules that facilitate glucose uptake in insulin resistant conditions whereas the secretagogues increases the total amount of insulin secretion from the beta-cells of the pancreas. Hypoglycemic agents such as sulfonylureas and biguanides (metformin) are well known examples of secretagogues and sensitizors respectively (Aguilar-Bryan L, et al., Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. Science 268:423-426, 1995; Lubbos H, et al., oral hypoglycemic agents in type II diabetes mellitus. American Family Physician. 52:2075-2078, 1995). The side effects of sulfonylureas include hypoglycemia, renal and hepatic disease, gastrointestinal disturbances, increased cardiovascular mortality, dermatological reactions, dizziness, drowsiness and headache. Biguanides lower blood glucose levels by reducing intestinal glucose absorption and hepatic glucose, but not by stimulating insulin secretion. The major side effects of biguanidine are lactic acidosis and increased cardiovascular mortality.

The other group of molecules that has immense potential are insulin mimetics and alpha-glucosidase inhibitors. The efficiency of the insulin mimetics are defined by the mimicking potential of unknown extracts where the activity is measured as the glucose uptake in differentiated 3T3 cells exposed to the test material only in absence of insulin. Alpha glucosidase inhibitors inhibit intestinal alpha glucosidases and consequently delay the digestion of sucrose and complex carbohydrates. The side effects of alpha glucosidase inhibitors include gastrointestinal side effects and hypoglycemia.

Objects and Summary of the invention:

The object of the present invention is to develop a multi – modal therapeutic approach for all degenerative diseases degenerative diseases like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye.

Future therapeutic strategies require the combination of various types of multiple agents. There is an urgent need to identify indigenous natural resources, procure them and study them in detail and their potential on newly identified targets in order to develop them as new therapeutics. Phytochemicals identified from traditional medicinal plants present an exciting opportunity to develop new kinds of therapeutics. Thus there is a need for a rationally designed interdisciplinary research programme, which could lead to the development of indigenous, renewable medicinal plant sources as practical and cost effective alternatives.

Medicinal preparations from herbal plants contain a variety of herbal and non-herbal ingredients that are believed to act on a variety of targets through various modes and mechanisms. The beneficial multiple activities like manipulating carbohydrate metabolism by various mechanisms, preventing and restoring integrity and function of beta cells, insulin releasing activity, improvement of the glucose uptake, utilisation and the anti oxidant properties present in the medicinal plants, which offers exciting opportunities, to develop them into novel therapeutics. The theories of poly herbal formulation have the synergistic, potentiative, agnostic / antagonistic pharmacological agents within themselves due to the incorporation of plant medicines with diverse pharmacological actions. These pharmacological principles are known to work in a dynamic way to produce maximum therapeutic efficacy with minimum side effects. Traditional medicines ought not to be treated as a collection of therapeutic recipes. They

are formulated and prepared, keeping in mind, the disease/sickness and the healing properties of the individual ingredients.

According to the present invention, etiology based cell assays and biochemical systems for the screening of plant extracts for anti-diabetic activities and degenerative disorders has been developed by developing formulations based on single or multiple extracts where the active constituents have been minimised to the least number that is required to retain activity.

The extracts and formulations prepared there-from addresses specific etiologies with scientifically validated leads for toxicity or efficacy from plants for the treatment of all degenerative disorders like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye.

The present invention is not to be limited in scope by the specific illustrations to the manifestations of diabetes but also to other degenerative disorders like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye..

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

The assays done include – i) 3H deoxyglucose uptake by 3T3-L1, ii) MTT assay and iii) Alpha-glucosidase assay.

i) The de-oxyglucose uptake assay by fat cells or adipoctes (3T3-L1) help to identify the bioactives that helps to increase the glucose uptake by the fat cells. These extracts will help the diabetic individuals by quick removal of glucose from blood stream and simultaneously will help the fat cells to build a better storage of energy.

ii) The MTT assay is a measure of cellular respiratory physiology. It can estimate the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

iii) Alpha glucosidase inhibitors inhibit intestinal alpha glucosidases and consequently delay the digestion of sucrose and complex carbohydrates. The presence of molecules of this type in the bio-actives will ensure a safe uptake of sucrose added products to the diabetics by delaying the intestinal glucose uptake.

As of now, 11 leads from 10 different plants using the cell based assays to have functions of insulin mimetics, insulin sensitisors, cellular respiration boosters and alpha-glucosidase inhibitors. All these assays were screened for further in-vitro assays e.g. glucose metabolism by hepatocytes, glucose uptake by myocytes and insulin secretion from the pancreatic cells.

The present invention is not to be limited in scope by the specific embodiments and examples, which are intended as illustrations of a number of aspects of the invention and any embodiments which are functionally equivalent are within the scope of this invention. Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the claims.

Brief description of the tables and figures:

Figure 1 illustrates the HPLC profile of 20% ethanol extract of Eugenia jambolana at various wavelengths to explain the process of complete metabolite profiling.

This process has been followed for all the extracts that we are discussing here. The actual measurements are done at 50 nm apart, here for easier representation we have only included data recorded at 200, 300, 400, 500, 600 and 700 nm.

Figure 2 illustrates HPLC profile of the insulin mimetic at 270 nm as expressed in Table 8.

Figure 3 illustrates the data from Table 8 expressed as a percent of the counts observed in the cells + insulin control.

Figure 4: HPLC profile of insulin sensitizors at 270 nm as expressed in Table 9.

Figure 5: The data from Table 9 are expressed as a percent of the counts observed in the cells + insulin control

Figure 6: HPLC profile of four medicinal plant fractions that showed significant α-glucosidase inhibitory activities in the screening procedure.

Figure 7: Shows that four medicinal plant fractions showed significant α-glucosidase inhibitory activities in the screening procedure.

Table 1: List of plants and their codes

Table 2 : Table of Solvents used in the experiments and their codes

Table 14: Screening of successive extracts of *Eugenia jambolana & Cinnamomum zeylanicum* and the Counts per minute observed in study of insulin mimetic activity (without insulin) and study of insulin sensitizing activity (with insulin)

Table 15: Screening of successive extracts of *Emblica officinalis & Catharanthus roseus* and the Counts per minute observed in study of insulin mimetic activity (without insulin) and study of insulin sensitizing activity (with insulin)

Detailed description of the Invention

We have screened plants as described in Table 1 using solvents as described in Table 2. We have done both direct parallel and successive extraction for the plants as described in Table 1. We have screened a majority of the plant extracts for their efficiency against degenerative diseases. The present invention relates to the function of multiple plant extracts that have been validated for specific anti-diabetic usage using multiple etiology based cell assays. It also relates to any future preparation of any number of compositions for degenerative diseases like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye and having antioxidant action, a process for the preparation of the same, and usage of the same. The invention also relates, more particularly, to any composition having as a major component a single extract or multiple extracts containing therapeutically relevant bio-actives as characterized here in Tables 4, 8, 9, 10, 11 & 12, obtained from plant/plants as described in Tables 1, 2 and 3 and being capable of lowering or reducing a level of glucose in the blood of a patient suffering from diabetes mellitus by alleviating or ameliorating various symptoms caused by or associated with the diabetes mellitus as well as preventing the glucose level in the blood of the diabetic patient.

This application claims benefit of priority 35 U.S.C 119 to PCT application no. PCT/IB03/03861 filed on 18th august 2003 and is incorporated herein in its entirety by reference.

A comprehensive review of the literature and documented evidence concerning the usage of medicinal plants and plant parts in the treatment of degenerative disorders will result in a list of greater than 200 plant species that have been reported to have varying degrees of efficiency. A meaningful screening procedure that would involve all the plants documented to have antidegenerative potential will require a huge allocation of resources, which in turn would make the entire process unviable. To circumvent this problem,

ADePt ™ was used during the lead selection process to arrive at a smaller number of plants (less than 20) that are being subjected to bioactivity based screening procedures.

This information has allowed a rational selection of the plants and plant parts, which have been the immediate focus for the discovery of novel bio-therapeutics for the treatment of all degenerative diseases like cardiovascular diseases, Diabetes mellitus, Hypertension, All types of Cancers, Obesity & weight problems, Arthritis & pain, Alzheimer's disease, Multiple sclerosis & Autoimmune diseases, Asthma & Allergies, Osteoporosis & bone health, Parkinson's disease, Stress, Disorders & diseases of the eye.

The targeted screening procedures feature, a comprehensive metabolite profiling of multitudes of phyto-extracts facilitate the creation of a metabolite grid. Extensive comparative analyses of the individual plant species with the pre-existing drug and phyto-extract formulations in the market reveals the presence of both unique and common molecular constituents that can be used individually and in combination to accelerate the process of discovery of novel therapeutic formulations.

The extraction and resolution of components present in complex phyto-extracts is an area that has been plagued by a lack of standard operating procedures that permit adequate standardization and quantitative estimation of metabolites at a comprehensive level. As part of the ongoing research program at Avesthagen, standard operating protocols have now been setup for all the steps that precede the enzymatic and cell-based bioassay procedures that are aimed at the screening of the multi-component phyto-extracts.

In this context, algorithms that help to establish comprehensive metabolite fingerprints of multi-component phyto-extracts have been integrated into ADePt™. Currently, ADePt™ features metabolite fingerprints of approximately phyto-extracts, derived from 15 short-listed medicinal plants with documented anti-degenerative potential that are being screened for bioactive constituents. Following are the HPLC conditions used for the analysis different medicinal plant extracts.

Instrument: Waters 2695 Separation module with Millennium^{32} software.

Detector : Waters 2996 photodiode array.

Column : μBandapak C-18, 4.6x150mm column, 10μm particle size.

Solvent A : Acetonitrile

Solvent B : Methanol

Solvent C : Milli-Q water

The list of plants described in table 1 were subject to successive extraction, starting with Petroleum ether, followed by Benzene, Chlororform, Ethyl acetate, Acetone, Methanol and Water; as described by the fraction id nos. in Table 12.

Fig 1 shows an example of how an integrated metabolite fingerprinting is run for one 20% ethanol extract of Eugenia jambolana at various wavelengths

The basic investigational strategy for the search of an agent having anti-degenerative potential is based on either of the two indicatory factors for the disease i.e. insulin and glucose. Table 1 gives the list of plants which were used for the experiment, table 2 gives the list of solvents used for extraction, table 3 gives the plant part used for the extraction and Table 4 & Table 12 gives the list of extracts under experimentation. The cell-based assays selected to measure glucose metabolism are based on the following parameters: Glucose uptake and Glycogen synthesis

Examples of the efficacy of the extracts:

Example 1: Study of insulin mimetic activity

"[^{3}H]-deoxyglucose uptake by 3T3-L1 adipocytes" is the classic assay that measures the ability of bioactive lead candidates to improve insulin sensitivity in stimulation of glucose uptake by adipocytes. The assay can also be adapted to measure insulin-mimetic activity by measuring stimulation of glucose uptake by plant extracts in the absence of insulin.

In this study we are looking for molecules that have insulin mimetic activity. So we measured the uptake of 3H-de-oxyglucose in adipocytes by incubating them with plant extracts in the absence of insulin. Mature adipocytes derived from 3T3-L1 fibroblasts were incubated with plant extracts alone followed by [^{3}H]-deoxyglucose uptake assay as described in details under the methods.

Method:

3T3-L1-cell culture

3T3-L1 fibroblasts were cultured in DME-F12 media containing 10% FCS, NEAA, glutamine, antibiotics, anti-mycotics, etc., in an atmosphere of 5% CO2 at 37 °C. Fibroblasts were cultured up to confluency. Subcultures were done at three-day intervals with trypsin-EDTA solution treatment.

Maturation of the fibroblasts to adipocytes

Differentiation was induced by treating the cells with DMEM hi glucose (4.5 gm / litre) containing 0.5mmol/l 3-isobutyl-1-methylxanthine, 4 mg/ml dexamethasone, 1 mg/ml insulin and 10% FCS for 48 h. The cells were cultured in DMEM media with standard pre-described supplements for the next 6-10 days. Cells were used only when at least 95% of the cells showed an adipocyte phenotype by accumulation of lipid droplets.

3H-deoxyglucose Uptake

In the first set of assays (insulin control and the first 9 samples) cells were grown in 96 well plates, and assayed for 3H-deoxyglucose uptake. Briefly cells were treated with plant extract overnight and immediately before the assay got washed with Krebs-Ringer-HEPES Buffer [20 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.5 mM MgSO4] at 37°C for 30 minutes and then treated with insulin. Glucose uptake reaction was initiated by adding 0.1 mM deoxy-glucose containing deoxy-tritiated-glucose containing 12.2 kBq/mL. After 30 minutes of 37° C incubation, the reaction was terminated by adding 40 uM cytochalasin-B. Cells were washed 3 X with ice cold KRH buffer and solubilized with 100 µl of 10% Triton-X with Ca/Mg free PBS. Radioactivity

incorporated into cells was measured on a top count microplate scintillation counter. Results are being interpreted as mean ± SD of counts per minute.

Table 5 shows standardization of [^{3}H]-glucose uptake with insulin. As the basal counts were low, the assay design was changed to a 24-well format and the cell numbers increased from 100, 000 cells per well to 2 million cells per well. As expected, this resulted in a dramatic increase in [^{3}H] intake counts while maintaining a high level of sensitivity to insulin.

Table 8 shows [^{3}H]-deoxyglucose uptake by the adipocytes from 3T3-L1 cells treated with plant extracts in the absence of insulin. The two controls in this experiment were: (a) [^{3}H]-deoxyglucose uptake by cells in the absence of both insulin and plant extracts, and (b) [^{3}H]-deoxyglucose uptake by cells in the presence of insulin and absence of plant extracts.

It was found that four plant extracts; namely AVDB008Le04(20)bc6C01A00, AVDB008Le06bc6C01A00, AVDB006Se07bc6C01A00 and AVDB005Le07bc6C01A00 stimulated [^{3}H]-deoxyglucose uptake by 1.5 to 2-fold above that seen with insulin alone.

Example 2: Study of cell viability and respiratory physiology

Crude plant extracts solubilized in a "compatible" solvent such as dimethylsulfoxide (DMSO) often affect the viability of cells in culture when incubated for long periods. It is necessary to optimize the appropriate dilution and time of incubation of cells with plant extracts under which cell viability remains unaffected. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium salt, (MTT) 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. The resulting

intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

MTT assay to determine cell viability when incubated with plant extracts

Method:

3T3-L1-cell culture

3T3-L1 fibroblasts were cultured in DME-F12 media containing 10% FCS, NEAA, glutamine, antibiotics, anti-mycotics, etc., in an atmosphere of 5% CO2 at 37 °C. Fibroblasts were cultured up to confluency. Subcultures were done at three-day intervals with trypsin-EDTA solution treatment.

MTT assay

The assay was done in 96 well flat bottom TC plates where the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is added to the cells treated or untreated with the plant extracts. It gets reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

Example 2a: Study of standardization of the dilution of the plant extracts

3T3-L1 fibroblasts were incubated with different concentrations of stock concentration of plant extracts (neat - 50 µg dry extract dissolved in 300µl DMSO, and 1:2 and 1:4 dilutions of it) for 24 hrs. The viability of the cells was assessed by MTT cell assays. Table 6 shows that neat extracts gave maximal MTT colorimetric reading. The standardization study was done with selective extracts only.

Table 6 and 7 show the standardization of the optimal concentration of plant extract that retains cell viability by MTT assay. 3T3-L1 fibroblasts were incubated with different concentrations of stock concentration of plant extracts (neat - 50 µg dry extract dissolved in 300µl DMSO, and 1:2 and 1:4 dilutions of it) for 24 hrs. The viability of the cells was assessed by MTT cell assays. Table 6 shows that neat extracts gave maximal MTT colorimetric reading. The standardization study was done with selective extracts only.

Example 2b: Study of standardization of the time points of co-culture of the extracts with the cell line

3T3-L1 fibroblasts were incubated with stock concentration of plant extracts (neat - 50 µg dry extract dissolved in 300µl DMSO) for 24, 48 and 72 hrs. The viability of the cells was assessed by MTT cell assays. Table 7 describes the standardization of the time point of incubation of cells with the plant extract required in the assay while retaining cell viability by MTT assay. It shows that the optimal incubation time for most of the extracts was 24 hrs. This standardization study was also done with selective extracts only. The routine MTT assay was done on all extract fractions and extracts that showed no cytotoxicity were taken up for evaluation of 3H de-oxyglucose uptake test.

Example 2c: Study of the influence of the extracts in producing respiratory enzymes in the cell line

3T3-L1 fibroblasts were incubated with stock concentration of plant extracts (neat - 50 µg dry extract dissolved in 300µl DMSO) for 24. Table 10 shows the optical density of the cell lysate that produced the formazon crystals. The color intensity corresponded with the amount of formazon produced that is an indicator of respiratory enzymes generated.

Example 3: Study of insulin sensitizing activity

In this study stimulation of [^{3}H]-deoxyglucose uptake in adipocytes was measured by incubating them with plant extracts in the presence of insulin. Mature adipocytes derived from 3T3-L1 fibroblasts were incubated with plant extracts in the presence of insulin followed by [^{3}H]-deoxyglucose uptake assay as described in details under the methods of example 1.

Table 9 and Figs 4 and 5 shows the HPLC profile and activity of the three plant extracts enhanced [^{3}H]-deoxyglucose uptake by adipocytes in presence of insulin, compared to the uptake observed in cells plus insulin alone. Table 4 below shows the raw counts.

In Figure 5, below, the data from Table 5 are expressed as a percent of the counts observed in the cells + insulin control.

It is observed that three plant extracts; namely, AVDB006Se04(20)bc6C01A00, AVDB018Fr03bc6C01A00 & AVDB003Ro06bc6C01A00, stimulated [^{3}H]-deoxyglucose uptake by nearly 2-fold above that seen with insulin alone. This enhancement in uptake of [^{3}H]-deoxyglucose in the presence of insulin indicates that the plant extract activity results in increased sensitization of adipocytes to respond to insulin.

Example 4: Alpha-glucosidase inhibition assay

Postprandial hyperglycemia and hyperinsulinemia, which are implicated in Diabetes and Obese conditions, are expected to be diminished by the inhibition of poly and oligosaccharide digestion in the intestinal tract. Alpha-glucosidase inhibitors, which act as competitive inhibitors of intestinal alpha-glucosidases, can delay the digestion and subsequent absorption of blood glucose rises.

Method

Various concentrations of plant extracts (inhibitor) are pre-incubated with the enzyme before adding the substrate (PNPG). Alpha-Glucosidase activity can be measured in-vitro

by determination of the release of p-nitrophenol arising from the hydrolysis of p-nitrophenyl-alpha-D-glucopyranoside by alpha-Glucosidase. The colour developed by the release of PNP (p-nitro phenol) can be measured quantitatively in the spectrophotometer.

Several experiments were performed in which the concentrations of a-glucosidase at constant substrate concentration was varied and the concentration of substrate was varied at constant substrate concentrations. An enzyme concentration of 0.04U/ml and the substrate (PNPG) concentration of 4mM were selected for the screening assays.

Table11 & Figs 6 and 7 shows the inhibitory activity of various concentrations of a plant extract in the alpha-glucosidase assay. In this case the IC_{50} (concentration showing 50% inhibition) was determined to be 50 μg/ml.

It was found that four medicinal plant extracts AVDB006Se08bc6C01A00, AVDB006Se04(20)bc6C01A00, AVDB006Se06bc6C01A00 and AVDB006Se04bc6C01A00 showed significant alpha-glucosidase inhibitory activity in the screening procedure.

While the present invention has been particularly shown and described with reference to the preferred embodiment thereof, it will be understood by those skilled in the art that various changes in form and details may be effected therein without departing from the spirit and scope of the invention as defined by the appended claims.

Table 1: List of plants and their codes

Sr. No.	Plant Name	Plant ID
1	Aegle marmelos	001
2	Azadirachta indica	002
3	Catharanthus roseus	003
4	Curcuma longa	004
5	Emblica officinalis	005
6	Eugenia jambolana	006
7	Glycyrrhiza glabara	007
8	Gymnema sylvestre	008
9	Lagerstroemia speciosa	009
10	Momordica charantia	010
11	Morinda citrifolia	011
12	Ocimum sanctum	012
13	Pterocarpus marsupium	013
14	Stevia rebaudiana	014
15	Swertia chirata	015
17	Tinospora cordifolia	017
18	Tribulus terestris	018
19	Trigonella foenum gracum	019
20	Withania somnifera	020
21	Cinnamomum zeylanicum	021
22	Garcinia combogia	022
23	Camellia sinensis	023
24	Vanilla fragrans	024
25	Coccinia indica	025

Table 2 : Table of Solvents used in the experiments and their codes

Solvent name	Solvent ID
Acetone	01
Benzene	02
Chloroform	03
Ethanol	04
Hexane	05
Methanol	06
Petroleum ether	07
Water	08
Ethyl acetate	09
Acetonitrile	10

Table 3: Table of Plant part/ tissue used in the experiment and their codes

Plant Part / Tissue	Part / Tissue ID
Aerial Parts	AP
Bark	Ba
Flower	Fl
Fruit Whole	Fr
Fruit pulp	FP
Leaf	Lf
Pod	Po
Rhizome	Rh
Root	Ro
Seed	Se
Tuber	Tu
Whole Plant	WP

Table 4: List of fractions of the plant extracts subjected to cell assays and the Counts per minute observed in study of insulin mimetic activity (without insulin) and study of insulin sensitizing activity (with insulin)

Sample number	Fraction ID	CPM without Insulin	CPM with Insulin
1	AVDB006Se08bc6C01A00	3104.8	59317.3
2	AVDB006Se04(20)bc6C01A00	50002.9	90587.7
3	AVDB019Se08bc6C01A00	32995.3	40797.1
4	AVDB019Se04(20)bc6C01A00	35818	26256.8
6	AVDB008Le08bc6C01A00	49174.8	1836.9
7	AVDB008Le04(20)bc6C01A00	78730	41782.6
8	AVDB013Ba08bc6C01A00	39701.8	43449.2
9	AVDB013Ba04(20)bc6C01A00	40732.3	35926
10	AVDB002Ba08bc6C01A00	40406.1	41058.9
11	AVDB005Fr06bc6C01A00	54150.5	2718.5
12	AVDB003Ro06bc6C01A00	40569.8	71661.7
13	AVDB020Ro06bc6C01A00	51048.2	10421.6
14	AVDB019Se06bc6C01A00	41022	4662.6
15	AVDB006Se06bc6C01A00	45698.5	4318.3
16	AVDB002Le06bc6C01A00	29289.9	16257.4
17	AVDB018Fr06bc6C01A00	33852.6	3196.3
18	AVDB017Fr06bc6C01A00	45025.2	2564.8
19	AVDB003Ro03bc6C01A00	44878.3	380.4
20	AVDB020Ro04bc6C01A00	35140.5	11758.2
21	AVDB020Ro03bc6C01A00	25536.6	38342.5
22	AVDB019Seo04bc6C01A00	34922	31443.8
23	AVDB004Se04bc6C01A00	40097.2	54145.4

24	AVDB018Fr04bc6C01A00	5030.4	35612.5
25	AVDB011Fr04bc6C01A00	21884.3	22019.8
26	AVDB017Fr04bc6C01A00	30027.6	22528.9
27	AVDB019Se03bc6C01A00	32921.9	32578.4
28	AVDB019Se03bc6C01A00	36945	50268.6
29	AVDB006Se03bc6C01A00	8765.5	31362.3
30	AVDB011Fr03bc6C01A00	39609.9	29006.6
31	AVDB017Fr03bc6C01A00	43817.2	26515.9
32	AVDB005Fr03bc6C01A00	42664.5	46780.6
33	AVDB008Le06bc6C01A00	41279.6	747.7
34	AVDB006Fr03bc6C01A00	689.1	41018.5
35	AVDB020Ro07bc6C01A00	2475.9	20976.4
36	AVDB019Se07bc6C01A00	3198.7	24380.4
37	AVDB006Se07bc6C01A00	67493	21301.2
38	AVDB018Fr07bc6C01A00	3572.9	4940.8
39	AVDB008Le07bc6C01A00	30539.8	62764.2
40	AVDB002Le05bc6C01A00	2594.6	28254.4
41	AVDB020Ro02bc6C01A00	2539.8	10946.4
42	AVDB019Se02bc6C01A00	30864	32429.1
43	AVDB002Se02bc6C01A00	3750	36523.4
44	AVDB018Fr02bc6C01A00	3103.1	20013.5
45	AVDB008Le06bc6C01A00	62200	32112.7
46	AVDB002Le02bc6C01A00	2185	44102
47	AVDB020Le08bc6C01A00	89.2	26084.7
48	AVDB008Le04bc6C01A00	1651.6	24005
49	AVDB002Le03bc6C01A00	50451.2	27349.1
50	AVDB002Le04bc6C01A00	1128.7	37275.8
51	AVDB002Le07bc6C01A00	546.5	64153.8
52	AVDB018Fr03bc6C01A00	16783.8	70859.9
53	AVDB008Le02bc6C01A00	12940	54596.2

54	AVDB003Ro04bc6C01A00	1953.6	30342.6
55	AVDB008Le03bc6C01A00	16799.2	47831.4
56	AVDB013Ba04bc6C01A00	17385.3	22041.3
56	AVDB008Le09bc6C01A00	24436.1	56640.7
58	AVDB008Le01bc6C01A00	37726.5	32916.7
59	AVDB019Se09bc6C01A00	35199	33672.2
60	AVDB019Se01bc6C01A00	59363.1	38863.4
61	AVDB005Le07bc6C01A00	66332.8	28386.2
62	AVDB005Le05bc6C01A00	33880.3	52381.6
63	AVDB006Se05bc6C01A00	44015.3	7996.3

Table 5: Table showing standardization of [^{3}H]-glucose uptake with insulin.

Cells/well	Control	Insulin 1 nM	Insulin 10 nM	Insulin 25 nM	Insulin 100 nM
100,000	25.3	60.5	502	ND	1850
2,000,000	1559	ND	36562	38538	55258

Table 6: Standardization of the optimal concentration of plant extract that retains cell viability by MTT assay

Fr no. → / Dil ↓	1	2	3	4	5	6	7	8	9
Neat	.811*	1.188	.411	.412	.376	.311	.717	.698	.523
1:2	.628	.726	.323	.363	.372	.279	.457	.516	.547
1:4	.482	.519	.390	.304	.356	.301	.479	.396	.428

Table 7: Standardization of the time point of incubation of cells with the plant extract required in the assay while retaining cell viability by MTT assay

Fr no. → / Dil ↓	1	2	3	4	5	6	7	8	9
24 hrs	.811*	1.188	.411	.412	.376	.311	.717	.698	.523
48 hrs	.534	1.262	.332	.270	.304	.288	.525	.427	.375
72 hrs	.142	1.199	.218	.223	.287	.267	.392	.298	.403

* Optical density (O.D. at 490 nm) control values – a) fibroblast alone 0.195 and b) DMSO control 0.285.

Table 8: [^{3}H]-deoxyglucose uptake by the adipocytes from 3T3-L1 cells treated with plant extracts in the absence of insulin. (mimetic)

Sr. No.	Extract ID.	Counts per minute
1	AVDB008Le04(20)bc6C01A00	78730
2	AVDB008Le04(20)bc6C01A00	66332
3	AVDB006Se07bc6C01A00	67493
4	AVDB008Le06bc6C01A00	62200
5	Cells alone	1559
6	Cells + insulin	36561

Table 9: [^{3}H]-deoxyglucose uptake by adipocytes in presence of insulin, compared to the uptake observed in cells plus insulin alone.

Sr. No.	Extract ID	Counts per minute
1	AVDB006Se04(20)bc6C01A00	90587
2	AVDB018Fr03bc6C01A00	70859
3	AVDB003Ro06bc6C01A00	71661
4	Cells alone	1559
5	Cells + insulin	36561

Table 10: MTT assay:

Extract nos	O.D. at 490 nm
AVDB008Le04(20)bc6C01A00	1.188
AVDB008Le04(20)bc6C01A00	0.311
AVDB006Se07bc6C01A00	0.414
AVDB008Le06bc6C01A00	0.497
AVDB006Se04(20)bc6C01A00	0.087
AVDB018Fr03bc6C01A00	0.08
AVDB003Ro06bc6C01A00	0.131

Table 11: The inhibitory activity of various concentrations of a plant extract in the alpha glucosidase assay

No.	Extract ID	IC_{50} / Potency		$1/IC_{50}$
1	AVDB006Se08bc6C01A00	60 µg/ml	+++	17
2	AVDB006Se04(20)bc6C01A00	55 µg/ml	+++	18
3	AVDB006Se06bc6C01A00	60 µg/ml	+++	17
4	AVDB013Ba08bc6C01A00	300 µg/ml	++	3
5	AVDB013Ba08bc6C01A00	300 µg/ml	++	3
6	AVDB019(20)Se02bc6C01A00	>1000 µg/ml	-	1
7	AVDB019Se08bc6C01A00	>1000 µg/ml	-	1
8	AVDB008Le04(20)bc6C01A00	>1000 µg/ml	-	1
9	AVDB008Le08bc6C01A00	No activity	-	0
10	AVDB020Ro08bc6C01A00	No activity	-	0
11	AVDB020Ro04bc6C01A00	1000 µg/ml	+	1
12	AVDB002Le08bc6C01A00	175 µg/ml	++	6
13	AVDB017Fr04bc6C01A00	800 µg/ml	+	1
14	AVDB003Ro04bc6C01A00	No activity	-	0
15	AVDB011Fr04bc6C01A00	475 µg/ml	+	2
16	AVDB018Fr04bc6C01A00	500 µg/ml	++	2
17	AVDB019Se04bc6C01A00	No activity	-	0
18	AVDB008Le04bc6C01A00	No activity	-	0
19	AVDB006Se04bc6C01A00	37.5 µg/ml	+++	27
20	AVDB002Le04bc6C01A00	650 µg/ml	+	2
21	AVDB005Fr06bc6C01A00	425 µg/ml	++	2
22	AVDB003Ro06bc6C01A00	>1000 µg/ml	-	1
23	AVDB020Ro06bc6C01a00	>1000 µg/ml	-	1
24	AVDB019Se06bc6C01A00	>1000 µg/ml	-	1
25	AVDB011Fr06bc6C01A00	850 µg/ml	+	1

Table 12: List of plant extracts subject to successive extraction

Sr. No.	Nomenclature
1	AVDB008Lf7S07(100)A1
2	AVDB008Lf7S02(100)A1
3	AVDB008Lf7S03(100)A1
4	AVDB008Lf7S09(100)A1
5	AVDB008Lf7S01(100)A1
6	AVDB008Lf7S06(100)A1
7	AVDB008Lf7S08(100)A1
8	AVDB019Se7S07(100)A1
9	AVDB019Se7S02(100)A1
10	AVDB019Se7S03(100)A1
11	AVDB019Se7S09(100)A1
12	AVDB019Se7S01(100)A1
13	AVDB019Se7S06(100)A1
14	AVDB006Fp7S07(100)A1
15	AVDB006Fp7S05(100)A1
16	AVDB006Fp7S03(100)A1
17	AVDB006Fp7S09(100)A1
18	AVDB006Fp7S01(100)A1
19	AVDB006Fp7S06(100)A1
20	AVDB006Fp7S08(100)A1
21	AVDB005Fr7S07(100)A1
22	AVDB005Fr7S05(100)A1
23	AVDB005Fr7S03(100)A1
24	AVDB005Fr7S09(100)A1
25	AVDB005Fr7S01(100)A1
26	AVDB005Fr7S06(100)A1
27	AVDB005Fr7S08(100)A1
28	AVDB001Lf7S07(100)A1
29	AVDB001Lf7S05(100)A1
30	AVDB001Lf7S03(100)A1
31	AVDB001Lf7S09(100)A1
32	AVDB001Lf7S01(100)A1
33	AVDB001Lf7S06(100)A1
34	AVDB001Lf7S08(100)A1
35	AVDB010Fr7S07(100)A1
36	AVDB010Fr7S05(100)A1
37	AVDB010Fr7S03(100)A1
38	AVDB010Fr7S09(100)A1
39	AVDB010Fr7S01(100)A1
40	AVDB010Fr7S06(100)A1
41	AVDB010Fr7S08(100)A1

42	AVDB003Le7S07(100)A1
43	AVDB003Le7S05(100)A1
44	AVDB003Le7S03(100)A1
45	AVDB003Le7S09(100)A1
46	AVDB003Le7S01(100)A1
47	AVDB003Le7S06(100)A1
48	AVDB003Le7S08(100)A1
49	AVDB012Ap7S07(100)A1
50	AVDB012Ap7S05(100)A1
51	AVDB012Ap7S03(100)A1
52	AVDB012Ap7S09(100)A1
53	AVDB012Ap7S01(100)A1
54	AVDB012Ap7S06(100)A1
55	AVDB012Ap7S08(100)A1
56	AVDB013Ba7S07(100)A1
57	AVDB013Ba7S05(100)A1
58	AVDB013Ba7S03(100)A1
59	AVDB013Ba7S09(100)A1
60	AVDB013Ba7S01(100)A1
61	AVDB013Ba7S06(100)A1
62	AVDB013Ba7S08(100)A1
63	AVDB014Lf7S07(100)A1
64	AVDB014Lf7S05(100)A1
65	AVDB014Lf7S03(100)A1
66	AVDB014Lf7S09(100)A1
67	AVDB014Lf7S01(100)A1
68	AVDB014Lf7S06(100)A1
69	AVDB014Lf7S08(100)A1
70	AVDB018Wp7S07(100)A1
71	AVDB018Wp7S05(100)A1
72	AVDB018Wp7S03(100)A1
73	AVDB018Wp7S09(100)A1
74	AVDB018Wp7S01(100)A1
75	AVDB018Wp7S06(100)A1
76	AVDB018Wp7S08(100)A1
77	AVDB010Fr7S07(100)A2
78	AVDB010Fr7S05(100)A2
79	AVDB010Fr7S03(100)A2
80	AVDB010Fr7S09(100)A2
81	AVDB010Fr7S01(100)A2
82	AVDB010Fr7S06(100)A2
83	AVDB010Fr7S08(100)A2

Table 13: Validation of 3T3-L1 assay

Validation molecules	Doses	Incubation Time	Counts per minute
None	-	-	4622.34
Insulin	25 nM	15 min	48026.7
Insulin	25 nM	30 min	24699.9
Insulin	25 nM	60 min	28103.3
Glitazone	60 μg	48 hrs	7886
Glitazone	150 μg	48 hrs	15900.4
Glitazone	300 µg	48 hrs	47971.9
Glitazone + insulin	60 µg + 25 nM	48 hrs / 15 min	19590.5
Glitazone + insulin	150 µg + 25 nM	48 hrs / 15 min	113168

Table 14: Screening of successive extracts and the Counts per minute observed in study of insulin mimetic activity (without insulin) and study of insulin sensitizing activity (with insulin)

Extracts	Counts without insulin	Counts with insulin
None	4622.34	-
AVDB021Ba08bc6C01A00	16475.2	53583
AVDB006Fp7S07(100)A1	11983.6	21189.5
AVDB006Fp7S05(100)A1	23563.7	36229.8
AVDB006Fp7S03(100)A1	31149.3	39991.6
AVDB006Fp7S09(100)A1	36229.8	40132.4
AVDB006Fp7S01(100)A1	413365	69396.0
AVDB006Fp7S06(100)A1	28102.8	39429.8
AVDB006Fp7S08(100)A1	13292.3	19981.9

Table 15: Screening of successive extracts and the Counts per minute observed in study of insulin mimetic activity (without insulin) and study of insulin sensitizing activity (with insulin)

Extracts	Counts without insulin		Counts with insulin	
None	1975.1	1831.0	-	-
Insulin	-	-	33065.1	32352.28
AVDB005Fr7S07(100)A1	67490.1	66563.6	39933.1	38480.9
AVDB005Fr7S05(100)A1	39196.9	38197.0	52662.1	57709.2
AVDB005Fr7S03(100)A1	28311.7	13361.3	36229.8	39198.9
AVDB005Fr7S09(100)A1	9055.6	8425.7	37506	37269
AVDB005Fr7S01(100)A1	6671.7	6961.4	44696.2	42544.7
AVDB005Fr7S06(100)A1	2400.5	2716.5	34279	32727
AVDB005Fr7S08(100)A1	785.7	1011.4	39929	480959
AVDB003Le7S01(100)A1	40580.3	48645.2	60119	73759
AVDB003Le7S06(100)A1	28311.8	28205.2	31401.2	32590
AVDB003Le7S08(100)A1	8294.9	7622.6	39929	38096

Table 16: HPLC method used for the analysis of AVDB006Se04bc6C01A00, AVDB006Se04(20)bc6C01A00, AVDB006Se06bc6C01A00, AVDB008Le04(20)bc6C01A00, AVDB008Le06bc6C01A00 and AVDB003Ro06bc6C01A00.

Time	Flow (ml/min)	Solvent percentage				Curve
		A	B	C	D	
1	1.0	10	10	80	0	6
5	1.0	10	25	65	0	6
10	1.0	10	50	40	0	6
15	1.0	10	90	0	0	6
20	1.0	10	80	10	0	6
25	1.0	10	60	30	0	6
26	1.0	10	10	80	0	1

Table 17: HPLC method was used for the analysis of AVDB006Se08bc6C01A00

Time	Flow (ml/min)	Solvent percentage				Curve
		A	B	C	D	
1	1.0	10	0	90	0	6
5	1.0	15	0	85	0	6
10	1.0	20	0	80	0	6
15	1.0	25	0	75	0	6
20	1.0	35	0	65	0	6
25	1.0	60	0	40	0	6
26	1.0	10	0	90	0	1

Table 18: HPLC method used for the analysis of AVDB006Se07bc6C01A00

Time	Flow (ml/min)	Solvent percentage				Curve
		A	B	C	D	
1	1.2	10	10	80	0	6
15	1.2	50	10	40	0	6
25	1.2	90	10	0	0	6
30	1.2	10	10	80	0	1

Table 18: HPLC method was used for the analysis of AVDB005Fr07bc6C01A00

Time	Flow (ml/min)	Solvent percentage				Curve
		A	B	C	D	
1	1.2	10	20	70	0	6
15	1.2	50	20	30	0	6
25	1.2	80	20	0	0	6
30	1.2	10	20	70	0	1

Table 19: HPLC method used for the analysis of AVDB018Fr03bc6C01A00.

Time	Flow (ml/min)	Solvent percentage				Curve
		A	B	C	D	
1	1.2	10	20	70	0	6
15	1.2	10	50	40	0	6
25	1.2	10	90	0	0	6
30	1.2	10	10	80	0	1